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HPLC-DAD method for the quantitative determination of short-chain fatty acids in meconium samples

Justyna Dobrowolska-Iwanek[a,*], Ryszard Lauterbach[b], Hubert Huras[c], Paweł Paśko[a], Ewelina Prochownik[a], Michał Woźniakiewicz[d], Sabina Chrząszcz[a], Paweł Zagrodzki[a]

[a] Department of Food Chemistry and Nutrition, Jagiellonian University Medical College, 9 Medyczna St, Kraków, 30–688, Poland
[b] Department of Neonatology, Jagiellonian University Medical College, 23 Kopernika St., Kraków, 31-501, Poland
[c] Department of Obstetrics and Perinatology, Jagiellonian University Medical College, 23 Kopernika St., Kraków, 31-501, Poland
[d] Laboratory for Forensic Chemistry, Faculty of Chemistry, Jagiellonian University, 2 Gronostajowa St., Kraków, 30-387, Poland

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ABSTRACT

The purpose of this study was to develop and validate a high-performance liquid chromatography with a diode-array detector (HPLC-DAD) method for the determination of short-chain fatty acids (SCFAs) such as: acetic, propionic, butyric, valeric and isovaleric acids in meconium. Additionally, the proposed analytical procedure was used to determine SCFAs in meconium obtained from 17 neonates. All determined organic acids in meconium samples were separated with good resolution. The HPLC system was characterized by high precision (the coefficient of variance ≤ 2.5%), high determination coefficients of calibration curves ($R^2 > 0.997$) and low limits of detection (LOD) and quantification (LOQ). For all investigated organic acids, LOD ranged from 0.01 to 0.80 mmol/ kg and LOQ values ranged between 0.04 and 2.64 mmol/ kg. Recovery of the HPLC assay for individual analytes comprised the range of 90 ± 2 - 106 ± 2%. Concentrations of acids in meconium collected from healthy children varied in wide ranges (CV within the range 0.79–2.47%) and no regularity was found in the molar ratios of the individual acids. The validation parameters obtained in this study, made the HPLC-DAD method reliable and useful tool for the determination of short-chain fatty acids in meconium samples.

1. Introduction

Short-chain fatty acids (SCFAs) are mainly monocarboxylic, saturated, aliphatic acids containing from 1 to 6 carbon atoms per molecule [1]. These include acetic, propionic, butyric, valeric, succinic or caproic acid [2]. SCFAs are the main and final products of a fermentation process carried out by anaerobic bacteria living in the human large intestine [3]. The basic substrates in the fermentation process are polysaccharides that have not been digested by intestinal enzymes before. They include non-starch polysaccharides (NSPs), dietary fiber and resistant starch, prebiotics (e.g. inulin), oligofructose or sugar alcohols (sorbitol, mannitol) [2, 4]. The acetic, propionic and butyric acids together account for 95% of the content of all short chain fatty acids produced in the human colon [5], while they remain in a relatively constant molar ratio of 60:20:18, 60:25:10 or 60:25:15 [1, 6, 7]. In much smaller amounts, also other short-chain acids are formed, such as formic, succinic and capric acid [1].

Short-chain fatty acids play a key role in the proper functioning of the human digestive system [8]. SCAFs are involved in the regulation of intestinal pH, facilitate the absorption of certain minerals (calcium, iron, magnesium) in the distal part of the colon, and promote the development of beneficial bacterial microflora, which, on a competitive basis, inhibits the growth of pathogenic bacteria [8–10].

The properties of butyric acid are particularly well described. This compound exhibits trophic effects associated with the acceleration of healing and regeneration within the intestinal epithelium, and has local anti-inflammatory, immunoregulatory and antineoplastic properties, although the latter one have so far been confirmed only by in vitro tests [4, 11–14].

Short-chain fatty acids show not only local effects on the colon epithelium, but after passage from the intestines to the bloodstream, they can also bind to fatty acid receptors - GPR41, GPR43, OLFR78, located in peripheral tissues [2]. In this way, SCFAs are able to regulate some physiological processes occurring in the human body. These

* Corresponding author.
E-mail addresses: justyna.dobrowolska-iwanek@uj.edu.pl (J. Dobrowolska-Iwanek), ryszard@lauterbach.pl (R. Lauterbach), hhuras@su.krakow.pl (H. Huras), p.pasko@uj.edu.pl (P. Paśko), ewelina.gajdzik@uj.edu.pl (E. Prochownik), michal.wozniakiewicz@uj.edu.pl (M. Woźniakiewicz), pawel.zagrodzki@uj.edu.pl (P. Zagrodzki).

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include effects on the immune system response to pathogens, modulation of blood pressure or regulation of leptin level secretion, a hormone that is involved in the regulation of fatty resources in the body, blood glucose levels and in reducing appetite [2, 15–17].

The production of short-chain fatty acids depends on many factors, including quantitative and qualitative composition of intestinal microbiota and the availability of substrates for the bacterial fermentation process [18]. It seems, that the proper composition of the intestinal microflora has a significant impact on the proper functioning of the human body.

Colonization of the gastrointestinal tract by intestinal bacteria begins at birth, and the first two years of child life are of key importance in the process of the final formation of the intestinal microflora. Colonization of intestine is time dependent with enterobacteriaceae and streptococci predominating during the first 1–3 days after the birth, when concentrations of these organisms peak at 10^{11} cfu/g in feces [19]. Certain factors, including the type of delivery (natural or cesarian delivery), feeding - breastfeeding or formula milk, the need to administer drugs, especially antibiotics, the use of probiotics, as well as hygiene and the environment life, may have a significant impact not only on the composition of the bacterial consortium, but also on whole health of an individual [20].

The analysis of the data carried out by Sevelsted et al. from the Danish national registers covering the years 1977–2012 (data from about 2 million children) showed that children born via cesarian section are significantly more exposed to diseases such as: asthma, juvenile arthritis, leukemia, systemic connective tissue disorders, immune deficiencies and inflammatory bowel disease [21]. This may be related to the reduction of the number of beneficial bacteria of the genus *Bifidobacterium* and *Lactobacillus*, as well as the generally lower level of biodiversity of bacterial microflora in children born by cesarian delivery compared to children who were naturally delivered [22]. Meconium is the very first stool of a newborn baby and is normally passed for the first 1–5 h after birth [23]. It begins to form in fetus gastrointestinal tract between 12 and 16 weeks of pregnancy and accumulates until birth [24]. Meconium, unlike later feces has usually very dark green color and it is almost odorless [25]. It has a complex matrix composed of amniotic fluid residue, cellular debris like flaked cells of the skin and of digestive tract, gastrointestinal secretions and enzymes, lanugo, fatty material from the vernix caseosa, cholesterol and precursors of sterols, bile acids and salts, mucopolysaccharide blood group substances [26], proteins [27], sugars [28] as well as trace elements [29]. So far meconium has been used mainly in toxicological research. It has been successfully employed in monitoring fetal exposure to drugs and their metabolites (e.g. cocaine, opiates, alcohol, cannabinoids) [23, 27, 30], persistent organic pollutants [31], pesticides [32] inorganic contamination (e.g. heavy metals) [33] and antibiotic [34]. There has been scarce investigations devoted to the determination of short-chain organic acids in meconium samples [35, 36]. SCFAs in meconium were mainly determined using the gas chromatography method developed by Zijlstra et al. [37] with modification introduced by Höverstad et al. [38]. Nevertheless, this methodology required purification by vacuum distillation which is time-consuming. Similarly, other approaches developed for determination of SCFAs in stools samples require removing of proteins [39] or further derivatization [40].

Rasmussen et al. [35] found that the molar ratio of acetic, propionic and butyric acids in meconium is 89:5:5, but one should note that according to mean and standard deviations of the concentrations of investigated acids reported therein, their variability should be considered as high. In case of particular individuals the molar ratio of analytes might be significantly different.

In this study, the validation of the simple analytical procedure using acidic extraction followed by the HPLC analysis for determination of short-chain organic acids in meconium was performed. Moreover, described procedure was applied to evaluate concentration of organic acids in meconium samples collected from 17 neonates.

The analysis of meconium samples, particularly the determination of SCFAs, can broaden the knowledge about the impact of food components in maternal diet on fetal metabolism. This is especially important because of contradictory information in the available literature about the profile and quantity of SCFAs in meconium [35, 36, 39]. Additionally, the analysis of SCFAs in meconium could be of great value also in establishing SCFAs as new and potentially significant indicators of different disturbances in gastrointestinal tract such as bacterial meconium infection or propionic acidosis. Thus the results of the study may lead to developing new therapeutic strategies.

2. Materials and methods

2.1. Chemicals

The deionized water of 18 M Ω cm was obtained from Milli Ro & Q water purification system (Merck-Millipore, Billerica, MA, USA). Organic acids and other reagents were of analytical standard grade or gradient grade. Acetonitrile (ACN), acetic acid, propionic acid, butyric acid, adipic acid, valeric acid, isovaleric acid and perchloric acid (70%) were purchased from Sigma-Aldrich (Steinheim, Germany), while 98% diethylamine and 85% orthophosphoric acid were purchased from Merck (Darmstadt, Germany).

2.2. Sample collection

Meconium samples were collected from 17 healthy children born either via cesarian section ($n = 8$) or through vaginal delivery ($n = 9$) in the University Hospital in Kraków. The meconium samples were gathered immediately after the first new-born's defecation into sterile plastic vessels. The collected samples were then instantly put into the freezer and stored at -20 °C, to avoid changes in the SCFAs concentration. The study design was approved by the Bioethics Committee of the Jagiellonian University Medical College.

2.3. Meconium sample preparation

Sample preparation procedure was based on our previous experiments [41]. Before the extraction process, samples were dried in a forced-air drying oven at +40 °C. Next, the samples were ground and mixed manually (mini handheld homogenizer) prior to be aliquoted. Meconium aliquots, of average amount of 500 mg of dry weight each, were transferred into 10 mL plastic tubes. Then the content of each vessel was mixed with 2.5 mL of perchloric acid (0.15 M) and the tubes tightly twisted. The extraction procedure was divided into two steps. During the first step the samples were exposed to ultrasound energy at +40 °C, (Ultrasonic Bath, Power 400 W, ULTRON U-503, Poland) for 80 min. During the second step the samples were shaken for 60 min at room temperature. After the extraction, the extracts were centrifuged for 15 min at 5000 rpm at room temperature. The supernatant was then filtered with mixed cellulose esters membrane (MCE, 0.45 μm, Nantong FilterBio Membrane Co., China). The sample extracts were stored frozen at −20 °C until analysis.

2.4. HPLC instrument

The concentrations of organic acids were determined using a high-performance liquid chromatography system (Prominence, Shimadzu, Kyoto, Japan) consisting of a Shimadzu LC-20AD pump, liquid chromatography SIL-20A autosampler and SPD-M20A diode-array detector. The analytes were separated on a Synergi 4 u Hydro-RP 80A C18 column (250 × 4.6 mm, 4 μm) at 35 °C. The HPLC system operated at flow rate 1 mL/min in gradient mode by mixing (A) ACN with (B) phosphoric buffer (pH 2.36), prepared by adding 1.4 mL 85% phosphoric acid and 1 mL diethylamine to a volumetric flask (1 L) and diluting to volume with deionized water to the mark. Both solutions were

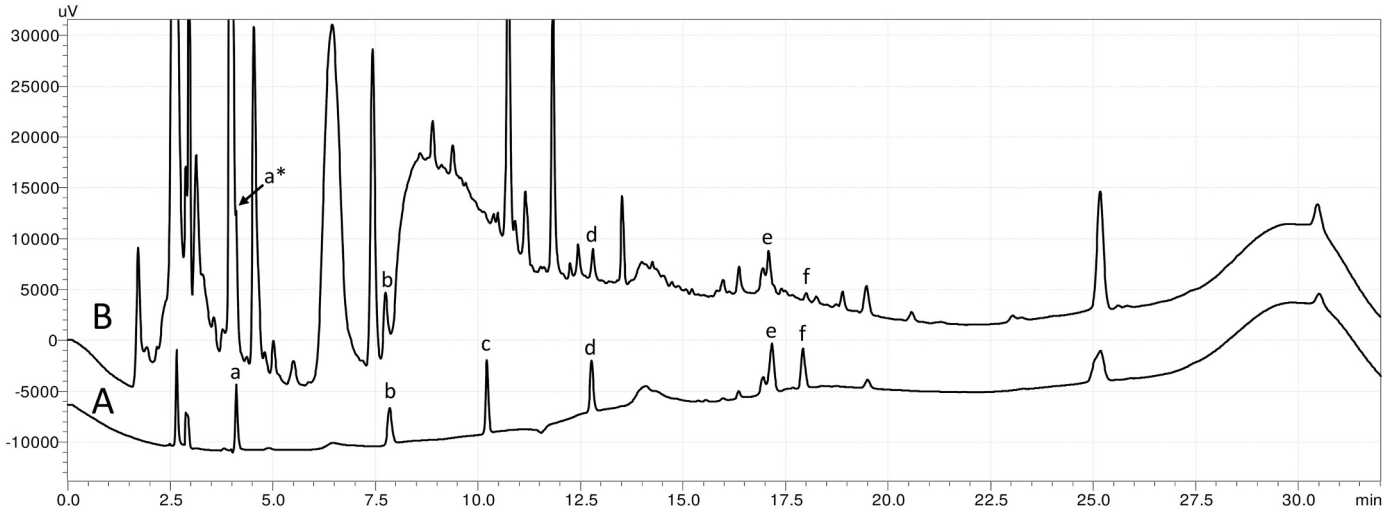

Fig. 1. Chromatographic separation (gradient elution) of organic acids in exemplary sample of meconium (chromatrogram **B**) and a standard mixture of six acids (chromatogram **A**). Signal assignments: a - acetic acid (a*- visible interference) , b - propionic acid, c - adipic acid, d - butiric acid, e - isovaleric acid, f – valeric acid.

additionally degassed by ultrasonication before use. The gradient program: 0 min A:5%, 6 min A:20%, 12 min A:30%, 16 min A:30%, 25 min A:40%, 28 min A:80%, 32 min A:80% and 35 min A:5% was used. After each run the column was further equilibrated for 3 min with A:5%. Such gradient program provide satisfactory resolution for all compounds of interest except acetic acid, and moreover the repeatability of retention times was below 1%. In this latter case a significant interference caused by matrix components was found, thus the determination of acetic acid was performed subsequently using mobile phase B for the isocratic elution. Each sample was analyzed two-fold (in three repetitions) using two different mobile phases. Before measurement, samples were appropriately diluted (5–25 fold) in deionized water (18 MΩcm) and filtered through a nylon 0.22-μm syringe filter (13 mm, Labe Ltd. Filter-Bio, Nantong, China). Samples were degassed in an ultrasonic bath.

2.5. Method validation

Validation was performed by evaluation of the following parameters of the method: linearity range, limits of detection and quantification, precision (CV) and accuracy calculated as the recovery of the HPLC assay. The peaks were identified basing on the retention time compared with ones collected within the analysis of standard mixture and by spiking samples with SCFAs standards to confirm the identification. For quantification of organic acids, the external calibration curve was calculated by the analysis of calibrators at following concentration levels: 3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/L for acetic acid, propionic acid, butyric acid, valeric acid and isovaleric acid. In addition, adipic acid was considered as non-characteristic of stool samples to test the selectivity of the method.

The linearity range was estimated by plotting peak area corresponding to each organic acid against the analyte concentration and then using the least squares method for calculation of respective regression coefficients and the determination coefficient (R^2). According to the validation protocol, the determination coefficient had to meet the criterion of $R^2 > 0.995$.

The precision of the method was expressed and the coefficient of variance and it was evaluated by successive analysis of the sample solutions, using six independent repetitions, at the same working day. The validation criterion for precision was ± 2.5%.

The recovery study was limited to evaluation of the HPLC assay, as the amount of meconium sample collected from newborns was highly limited. It was determined by spiking the sample extracts with known concentrations of analytes (+50% of an analyte amount determined in an initial measurement of the same but non-spiked sample).

The limit of detection (LOD) and limit of quantification (LOQ) for each organic acid were calculated by taking into account the residual standard deviation (SD) of the analytical signal and slope of the calibration curve (s), according to the formulas: LOD = 3.3 SD/s, LOQ = 10 SD/s, respectively.

2.6. Statistical analysis

Descriptive statistics were calculated for all fatty acids. Comparisons between concentrations of different fatty acids were performed using Kruskal-Wallis test with Dunn's post hoc test to reveal the differences between the paired acids. Differences with $p < 0.05$ were considered as statistically significant. The statistical analyses were carried out using packages STATISTICA v. 12 (Statsoft, Tulsa, OK, USA) and GraphPad Prism v. 3.02 (GraphPad Software, San Diego, USA).

3. Results and discussion

The method for determination of short-chain fatty acids in meconium was developed and subsequently validated. The chromatograms of the HPLC analysis of a standard mixture and the example of meconium sample are presented in Fig. 1. The proposed HPLC method allowed the separation of five short-chain acids in meconium samples with satisfactory resolution. The possible interference between the isovaleric peak (Fig. 1, peak f) and the adjacent peak was found negligible for quantitative and qualitative analysis. The peak purity evaluation revealed that the influence of the interference is low and stable and it does not affect the precision and accuracy of the method if peaks are splitted in the valley point and the base line is marked using the drop perpendicular methodology.

One should note, that due to analytical interferences acetic acid cannot be determined using gradient method, so in this case a different methodology was used (see HPLC instrument section) and results are demonstrated in Fig. 2. The validation parameters are presented in Table 1. Calibration curves for all analytes were linear in the concentration range of 3.125 - 200 mg/L with high determination coefficients of calibration curves ($R^2 > 0.997$). The HPLC system was characterized by high sensitivity and low limits of detection and quantification, particularly the lowest LOD and LOQ values were obtained for propionic acid, which were 0.01 and 0.04 mmol/kg respectively. The highest LOD and LOQ parameters were found for acetic acid and they were 0.80 and 2.64 mmol/kg, respectively.

Valerio et al. [42] evaluated the parameters of the method for

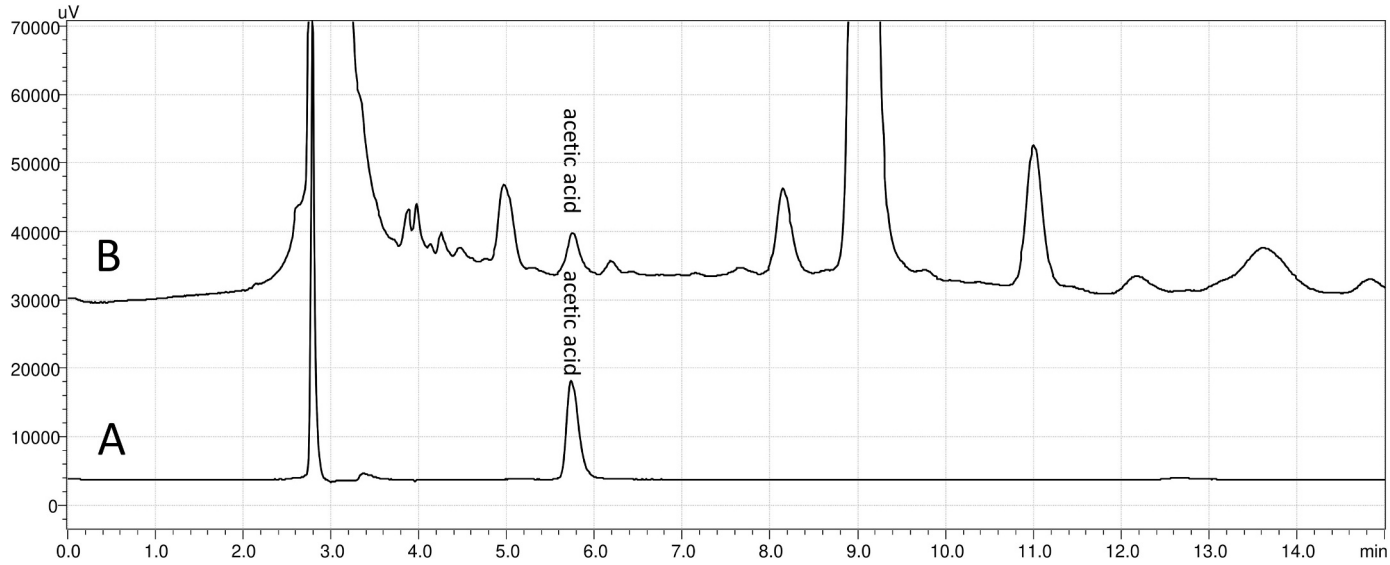

Fig. 2. Chromatographic analysis (isocratic elution) of exemplary sample of meconium (chromatrogram **B**) and a standard solution of acetic acid (chromatogram **A**); visible signal of acetic acid.

Table 1
Validation parameters for organic acids in meconium samples obtained using HPLC method.

Acids	Coefficient of determination (R^2)	LOD (mmol/kg)	LOQ (mmol/kg)	CV (%), $n = 6$	Recovery (%)
acetic	0.9985	0.80	2.64	1.80	106 ± 2
propionic	0.9999	0.01	0.04	1.75	101 ± 2
adipic	0.9971	0.65	1.95	2.47	105 ± 1
butyric	1.000	0.24	0.72	1.41	101 ± 1
isovaleric	0.9998	0.30	0.90	0.79	90 ± 2
valeric	0.9976	0.59	1.76	0.97	99 ± 1

determining the organic acids in adult fecal samples using the HPLC system with 3-channel UV detector. The limits of quantification for propionic and butyric acids were found to be higher (1.72 and 0.85 mmol/kg, respectively) and for acetic, valeric and isovaleric acids lower (0.55, 0.48 and 0.09 mmol/kg, respectively) than those reported in the present study. However, it should be noted that the established LOQs proved to be sufficient to determine the last three acids in all samples of the meconium in our study.

Recovery of the HPLC analysis for individual compounds comprised the range of 90 ± 2 -106 ± 2% ($n = 3$) which indicates that the developed HPLC separation is not affected by interferences. However, only the sample extracts were spiked with appropriate concentration of analytes and such approach confined the insight into the effect of sample preparation on recovery, but the limited amount (enough only for a single extraction) and number of available meconium samples forced such protocol. The results were characterized by high precision as CV determined for each acid did not exceed 2.5%. The performance parameters of SCFAs determination in meconium material using the HPLC method are not available in literature. It should be stressed that reference analytical procedures for SCFAs determination in stool samples were actually developed and used to determine these analytes in human feces and not particularly in meconium [35, 36]. It is well known that human fecal matter varies according to the diet and health status. Therefore, the chemical composition of feces of neonates (meconium) and of adults might be extremely different, except for water content as it water constitutes always 75–80% of the stools. In consequence, one may expect e.g. different interferences in case of the analysis of these two materials.

Despite some disadvantages, gas chromatography is still the most commonly used analytical method to determine meconium SCFAs. Rasmussen et al. [35] and Midtvedt et al. [36] measured short-chain fatty acids in meconium samples after acidification (with H_2SO_4) and vacuum distillation steps according to the method of Zijlstra et al. [37] with modification by Höverstad et al. [38]. Recoveries for the acids in the whole analytical procedure (including sample pretreatment) ranges from 90–109% , thus it was similar to those received in this work.

However, the pretreatment procedure [37, 38] is time consuming and therefore it is (i) associated with the risk of loss of volatile acids, and (ii) difficult to use for large number of samples (e.g. in routine analysis). Underwood et al. [40] determined also short-chain fatty acid in stool samples from infants at age of 4 weeks. The preparation of the sample for measurement consisted of several main stages, such as: acidification (with 0.15 mmol/L H_2SO_4), derivatisation (with acidic 2-nitrophenylhydrazine hydrochloride), extraction with hexane to remove long-chain fatty acids hydrazides and with diethylether to separate the SCFA derivatives, and finally evaporation of the solvent overnight. Obtained extracts were analyzed using reverse-phase HPLC-UV method. In turn, Szylit et al. [39] used mercuric chloride to remove proteins and phosphotungstic acid solution was added and settle overnight to deproteinize supernatants. SFCA separation was conducted by means of GC-FID. In both of the cited procedures, no method parameter was shown. In our work, the sample preparation for measurement was based on the previously developed method with some modification [41] consisting of three short steps: acidification with 0.15 M $HClO_4$, ultrasound digestion (80 min. at 35 °C) and shaking (60 min.). In comparison to the previous procedure, the duration of individual steps has been lengthened. Furthermore, the developed protocol is more efficient and less time consuming than those presented in the past. Indeed, the time required to accomplish such two steps analysis (as in the developed protocol, which involves two separate chromatographic runs), is longer, but still acceptable if compared to GC protocols, well established and widely used in fatty acids analysis, e.g. using the FFAP column (free fatty acid phase column) without derivatization of analytes (ca. 20 min) [43]. Nevertheless, according to some reports on FFAP columns, on the one hand – they offer really fast analyses and share certain characteristics of PGE columns, but on the other – they are also less stable and robust and have lower temperature limits than most polysiloxanes columns [44], which requires derivatization lasting from several minutes to 48 h [45].

The whole procedure for preparing the sample for the measurement is relatively quick and easy, however, it is associated with the risk of

Table 2
Concentration of organic acids in meconium [mmol/kg].

Sample	Concentration of organic acid in meconium [mmol/kg]					Molar ratio
	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid	Valeric acid	
1CS	49.83 ± 0.99	23.84 ± 1.37	9.14 ± 0.15	2.50 ± 0.08	3.64 ± 0.04	56:27:10:3:4
2CS	76.67 ± 1.68	10.77 ± 0.41	157.25 ± 6.13	9.96 ± 0.28	42.84 ± 2.17	26:4:53:3:14
3CS	58.17 ± 1.25	<LOD	17.73 ± 0.27	1.25 ± 0.00	4.10 ± 0.06	-
4CS	13.83 ± 0.31	5.81 ± 0.18	15.46 ± 018	2.49 ± 0.06	4.25 ± 0.37	33:14:37:6:10
5CS	23.17 ± 0.23	66.26 ± 3.40	195.81 ± 3.51	37.26 ± 2.49	69.01 ± 2.10	6:17:50:9:18
6CS	22.50 ± 0.45	3.65 ± 0.04	6.19 ± 0.33	1.85 ± 0.02	2.99 ± 1.16	60:10:17:5:8
7CS	92.33 ± 1.71	<LOD	61.54 ± 0.93	11.04 ± 0.54	18.64 ± 1.13	-
8CS	124.17 ± 2.26	<LOD	83.28 ± 0.50	35.58 ± 1.14	57.68 ± 0.16	-
1VD	24.5 ± 0.35	28.57 ± 0.11	1.31 ± 0.04	2.40 ± 0.07	7.76 ± 0.04	38:44:2:4:12
2VD	56.42 ± 1.2	26.49 ± 3.56	133.34 ± 3.35	26.04 ± 0.53	41.81 ± 3.31	20:9:47:9:15
3VD	33.92 ± 0.55	4.75 ± 0.24	170.25 ± 1.21	11.22 ± 0.13	32.79 ± 0.67	13:2:67:5:13
4VD	36.75 ± 0.48	<LOD	67.62 ± 0.45	6.06 ± 0.06	28.48 ± 0.42	-
5VD	47.94 ± 0.92	<LOD	25.02 ± 0.80	5.38 ± 0.04	11.83 ± 0.24	-
6VD	40.17 ± 0.79	<LOD	14.40 ± 0.22	7.53 ± 0.09	10.37 ± 0.06	-
7VD	21.04 ± 31	<LOD	230.24 ± 8.05	4.98 ± 0.18	98.22 ± 0.17	-
8VD	40.00 ± 0.77	<LOD	14.57 ± 0.09	1.96 ± 0.18	3.42 ± 0.20	-
9VD	35.34 ± 0.91	121.23 ± 7.70	110.52 ± 0.62	28.47 ± 0.72	24.21 ± 1.06	11:38:35:9:7
Median*	**40.00^{ab}**	**3.65^{ac}**	**61.55^{cd}**	**6.06^{bd}**	**18.64**	

CS - Meconium samples from children born via cesarian section.
VD - Meconium samples from children born via natural vaginal delivery.
a. b. c. d - the same letters indicate significant differences in median values ($p < 0.05$).
* In case of propionic acid median was calculated taking into account substitution of <LOD results with LOD/√2 = 0.0007.

impurities, that can cause overloading of the system and column contamination. As a consequence, it may affect the quality of the chromatographic column and worse the separation of sample analytes. In order to prevent this, a pre-column was used and after each batch of analyzed samples the column was rinsed for a long time with water and acetonitrile (20:80). During the study, no significant changes in retention time nor peak shapes were observed.

3.1. Application of the method

Meconium samples were collected from 17 healthy children born either via cesarean or vaginal. None of the samples showed the presence of adipic acid. The determined values of concentrations of all acids were expressed in mmol per kg of dry matter (Table 2.). In case of 10 samples, the concentration of propionic acid was below LOD. In order to include all observations in calculations of descriptive statistics (and thus into Median value in Table 2) the <LOD results were substituted with LOD/√2, according to the method described in [46]. It was noticed that the concentrations of acids in meconium varied in wide ranges, and, unlike in adult human feces, there was no clear trend in the case of molar ratios of individual acid concentrations. Each stool sample had a different ratio of short-chain organic acids. Fig. 3. shows the

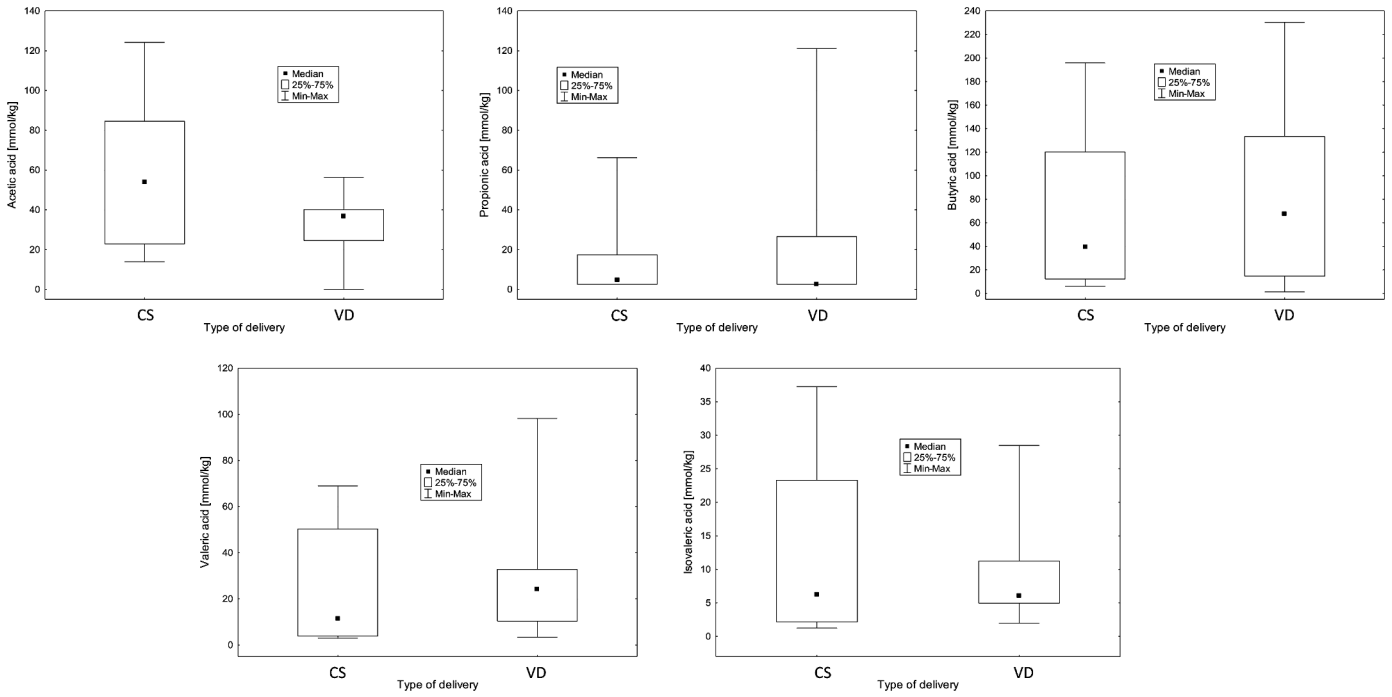

Fig. 3. The median concentrations of SCFAs in meconium from children born either via cesarean section (CS) or vaginal delivery (VD).

comparison between the median concentrations for individual acids in the meconium samples of infants born via cesarian section (CS) or vaginal delivery (VD). Due to the small number of samples within one group, statistical analysis was not performed. Acetic acid (median: 40.00 mmol/kg) and butyric acid (median: 61.55 mmol/kg) were the most abundant SCFAs found in meconium samples. The median concentrations of these SCFAs were significantly higher than determined propionic (median: 3.65 mmol/kg) and isovaleric acids (6.06 mmol/kg) ($p < 0.05$). The differences between the median values of acetic acid and butyric acid and of propionic and isovaleric acids were not statistically significant. The mean concentration value determined for valeric acid and other acids did not differ significantly.

Midtvedt et al. [36] studied the changes in short-chain fatty acid concentrations in feces from 29 healthy children during the first two years of their life. In the meconium of all children, acetic acid was determined at mean value of 5.2 mmol/kg. Only in case of four investigated children meconium samples propionic acid (mean value: 0.1 mmol/kg) and *n*-butyric acid (mean value: 0.5 mmol/kg) were quantitatively estimated. Other investigated SCFAs, such valeric and *i*-valeric acids as well as and *i*-butyric and *n*-caproic acids were not found in meconium samples.

In our study we determined acetic acid, butyric acid, valeric acid and *i*-valeric acid in every sample, and propionic acid in 10 samples. Considering that the water content in meconium is about 80%, the obtained median concentrations of acetic, n-butyric and propionic acids (40.00 mmol/kg, 61.55 mmol/kg and 3.65 mmol/kg, respectively) in the tested material were higher than those obtained by Midtvedt et al. In turn, Rasmussen et al. [35] determined the concentrations of acetic, propionic and butyric acid in meconium from 13 newborns. The acetic acid was the most abundant one, while the concentration of propionic and butyric acids was much lower. However, due to the ambiguous interpretation of units in which the concentration of analytes (mmol/L) was expressed the results obtained by Rasmussen et al. cannot be directly compared with results presented in this work. Moreover, the variability of the SCFAs concentrations in meconium samples, reported by Rasmussen et al. [35], suggests that the actual SCFAs molar ratio may vary between children as it was also found in our study (see Table 2).

It should also be noted that the meconium itself is not a homogeneous sample - its formation begins around the 20th week of fetal life and continues until delivery. Therefore, the samples of meconium taken for testing may differ in their qualitative and quantitative composition. Another reason for the differences in the SCFAs concentration may be the disproportions in the gut composition of the intestinal microflora of children. It may be influenced by such factors as: latitude of the research, hygienic conditions in the hospital, where the child was born, a labor type (cesarian section / vaginal delivery). Additionally, the above mentioned study did not take into account the impact of prenatal factors, such as maternal health, medicines used during pregnancy, diet and lifestyle, or socioeconomic conditions.

4. Conclusions

In summary, because of the shortage of work considering the influence of the mode of delivery on the content of short-chain fatty acids in meconium of newborns, it is advisable to conduct further experiments in this field, even on a larger scale (taking into account other factors such as the child's weight or duration of pregnancy or different diseases). The parameters obtained during the validation process indicate that the developed method using HPLC with the DAD detector is reliable, therefore, it can be used to analyze meconium. It is expected that through increasing number of objects and taking into consideration other factors in multivariate statistical analysis, we will better understand the role and source of the SCFAs in newborns' gastrointestinal tract.

CRediT authorship contribution statement

Justyna Dobrowolska-Iwanek: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Visualization, Supervision. **Ryszard Lauterbach:** Investigation, Resources. **Hubert Huras:** Investigation, Resources. **Paweł Paśko:** Conceptualization, Investigation, Writing - original draft. **Ewelina Prochownik:** Investigation. **Michał Woźniakiewicz:** Conceptualization, Writing - original draft, Supervision. **Sabina Chrząszcz:** Investigation. **Paweł Zagrodzki:** Formal analysis, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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